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# DNA Demethylation In Vitro: Involvement of RNA

**Alona Weiss, Ilana Keshet, Aharon Razin, and Howard Cedar**
Department of Cellular Biochemistry
The Hebrew University Medical School
Jerusalem 91120
Israel

## Summary

**An in vitro system for studying DNA demethylation has been established using extracts from tissue culture cells. This reaction, which is unusually resistant to proteinase K, takes place through the removal of a 5-methylcytosine nucleotide unit from the DNA substrate and its conversion to an RNase-sensitive form. It is likely that this represents the in vivo mechanism, as well, since extracts from L8 mybolasts specifically demethylate an α-actin gene, while extracts from F9 teratocarcinoma cells specifically demodify the *Aprt* CpG island. After pretreatment with proteinase K, these extracts demethylate both genes equally, suggesting that gene specificity may be controlled by protein factors.**

## Introduction

The animal genome is characterized by a bimodal methylation pattern that is correlated with gene expression. Housekeeping genes contain 5′ CpG islands, which are constitutively unmethylated (Bird, 1986), while cell type–specific genes are modified in every tissue except in their tissue of expression (Yeivin and Razin, 1993). This basic pattern is generated anew in each individual during development by means of a dynamic process involving both de novo modification and demethylation. Soon after fertilization, methyl moieties derived from gametic DNA are removed from almost all CpG residues in the genome, and this overall unmethylated state is then maintained through the blastula stage (Monk et al., 1987; Chaillet et al., 1991; Kafri et al., 1992). Establishment of the basic somatic bimodal methylation pattern occurs at about the time of implantation with the induction of a de novo methylation activity (Jahner and Jaenisch, 1984) that remodifies most of the CpG sites, but leaves the islands unmodified (Kafri et al., 1992). This process is carried out by an embryo-specific demethylation reaction (Frank et al., 1991) that preferentially recognizes CpG islands by virtue of the Sp1 elements present in these sequences (Brandeis et al., 1994; Macleod et al., 1994).

Once this ground-state methylation pattern is set up in the early embryo, it is maintained throughout development by a maintenance methylase that preserves the fixed positions of methyl moieties from cell generation to generation following DNA replication (Pollack et al., 1980; Wigler et al., 1981). Postimplantation de novo modification is probably one of the most critical steps in embryogenesis, since it organizes the genome into a fixed expression pattern whereby tissue-specific genes are globally repressed and housekeeping genes are active in every cell type. Indeed, embryos that are deficient for the DNA methyltransferase gene are severely defective and only survive for a few days after implantation (Li et al., 1992).

While this basic methylation pattern is generally preserved throughout development, additional local demethylation events take place on individual genes as part of the activation process associated with tissue-specific differentiation. The end result is that each cell-type specific gene is modified and repressed in most tissues, but is unmethylated in the tissue of expression. This demethylation is a programmed part of the developmentally regulated gene activation mechanism and is controlled by *cis*- and *trans*-acting factors. In the case of the muscle-specific α-actin gene, for example, *cis*-acting sequences in the upstream regulatory region are required to direct the local demethylation that takes place exclusively in myoblast cells (Paroush et al., 1990). In a similar manner, the IgG κ-chain gene undergoes specific demodification in B cells in a reaction that is driven by a cluster of *cis*-acting sequences that include the intronic κ enhancer and the nearby matrix attachment region (MAR) element (Lichtenstein et al., 1994). For both of these genes, methyl moieties inhibit transcription in their specific cell types, and demethylation must therefore take place prior to the expression of the gene. These studies thus suggest that demethylation is an important step for the activation of tissue-specific genes during organogenesis and that this process is most likely carried out by a combination of *trans*-acting factors that recognize *cis*-regulatory elements in the vicinity of the gene.

It has been suggested that demethylation in vivo takes place through a passive mechanism whereby the maintenance methylase is inhibited at specific sites. In this scenario, full double-stranded demethylation would require at least two cell divisions and would occur in only 50% of the cells. Although this type of demodification does take place following treatment of cells with 5-azacytidine (Jones, 1984), there is as yet no evidence that this is the mechanism in vivo. Indeed, in several instances it has been proven that demethylation takes place through an active mechanism. Mouse erythroleukemia cells, for example, undergo a genome-wide transient demethylation in response to treatment by hexamethylene bisacetamide (HMBA). By carefully monitoring the kinetics of this reaction, it was shown that methyl groups are removed at times in the cell cycle when DNA is not even undergoing replication (Razin et al., 1986). Gene-specific demethylation must also occur by an active mechanism, since both δ-crystallin (Sullivan and Grainger, 1986) and vitellogenin (Saluz et al., 1986) become demethylated in their tissues of expression even while DNA synthesis is inhibited. Perhaps the best evidence for an active mechanism comes from studies of the α-actin gene transiently transfected into L8 myoblasts (Paroush et al., 1990). In this system, full demethylation occurs on unintegrated prelabeled plasmid molecules, and this would have been impossible in a passive mechanism.

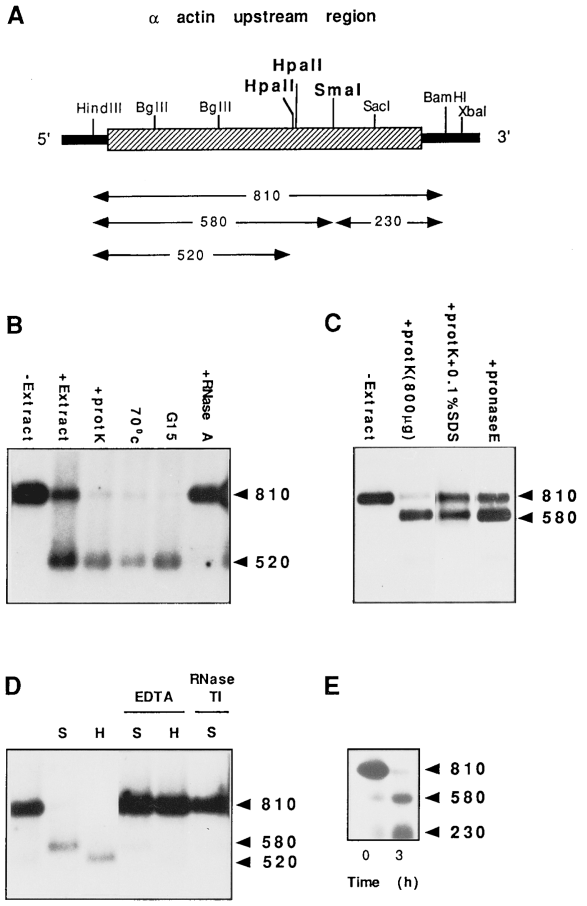


Figure 1. In Vitro Demethylation Assay

The end-labeled in vitro methylated α-actin HindIII–BamHI gene fragment was added to cell extract preparations from L8 myoblasts. After incubation at 32°C, the DNA was purified, denatured, and rehybridized to an excess of the unlabeled, unmethylated pBSα–act plasmid cut with HindIII–BamHI. The resulting DNA was then tested for DNA methylation by digestion with HpaII or SmaI and electrophoresis on 1% agarose gels (see Experimental Procedures).

(A) A map of the α-actin upstream region from the pBSα–act plasmid showing the appropriate restriction sites and the fragment sizes expected from digestion by HindIII–BamHI together with HpaII or SmaI. The closed region represents the plasmid polylinker. SacI and BglII sites were used in the experiments described in Figure 4. HpaII methylase modifies this fragment at three sites, including the SmaI locus (a subset of the HpaII recognition sequence).

(B) The in vitro methylated α-actin fragment was end labeled at the HindIII site, incubated with various extract preparations for 15 hr, and tested by cleavage with HpaII. Gel analysis shows the degree of methylation without extract, in the presence of untreated extract, using extract treated with proteinase K, extract pretreated for 10 min at 70°C, extract following G15 column chromatography and isolation of the void volume, or extract pretreated with 100 μg/ml RNase A for 1 hr at 37°C. It should be noted that proteinase K pretreatment for 1 hr at 56°C had no detrimental effects on the demethylation reaction, even if the extract was first diluted 10-fold (data not shown). In addition, full demethylation was observed after heat treatment at 70°C for 10 min on either untreated or proteinase K–pretreated extracts and after G15 chromatography. After heating to 95°C under the same conditions, about 30% of the activity remained. Some demethylation activity was also retained after the extracts were treated with phenol and the reaction was allowed to continue in the aqueous phase.

(C) A separate cell extract was pretreated for 1 hr at 56°C with either 800 μg/ml proteinase K or 200 μg/ml proteinase K in the presence of 0.1% SDS or with 300 μg/ml pronase E (Sigma) at 37°C. These preparations were then reacted with end-labeled fragment for 3 hr, and activity was determined by digestion with SmaI. Experiments using the same extract treated with 200 μg/ml proteinase K in the presence of 0.1% sarkosyl (Sigma) also yielded about 50% demethylation.

(D) The same methylated fragment was incubated with a different proteinase K–pretreated extract preparation for 3 hr and tested for demethylation by cleavage with HpaII (H) or SmaI (S). The extract was also tested for activity in the presence of 10 mM EDTA or after pretreatment with RNase $T_1$. The first lane shows the same substrate at time zero, cut with HpaII.

(E) The in vitro methylated α-actin fragment was end labeled at both the HindIII and BamHI sites and incubated with a proteinase K–pretreated extract for 0 or 3 hr and tested for demethylation by cleavage with SmaI.

Very little is known about the actual biochemical mechanism of demethylation. In principal, this could take place by removing either the methyl moiety itself, the methylated cytosine base, or the entire nucleotide. The removal of uracil or other aberrant bases from DNA usually takes place in a stepwise manner involving glycosylation and local DNA repair (Lindahl, 1982), and this may also be the mechanism by which T-G mismatches are corrected in animal cells (Wiebauer and Jiricny, 1990). Experiments in erythroleukemia cells showed that 5-methylcytosine residues are replaced with labeled cytosine during the demethylation reaction, suggesting that the target of this reaction is either the base alone or the full nucleotide (Razin et al., 1986).

We have now developed an in vitro system for studying demethylation, enabling us to dissect the substrates and products of this chemical process. Our results show that this reaction is at least partly mediated by RNA molecules and that demethylation takes place through the removal of DNA nucleotides and their conversion to an RNase-sensitive form. Extracts from different cell types catalyze demethylation in a gene-specific manner, strongly suggesting that this activity is the same as that used in vivo during normal development.

## Results

### Demethylation Assay

Since the rat α-actin gene undergoes active demethylation when transiently transfected into L8 myoblasts (Paroush et al., 1990), we developed an in vitro assay for demodification based on this system. The substrate, an 810 bp fragment from the upstream region of the gene, was methylated in vitro at three sites using HpaII methylase, end labeled, and then exposed to a cell-free extract derived from the same cells (Figure 1A). When modified, this fragment cannot be cut by the restriction enzymes SmaI or HpaII, but following demethylation the sites become cleavable. These enzymes do not digest hemimethylated DNA, however. Thus, to make the assay more sensitive, we denatured the reaction products and reannealed them to a large excess of unmethylated carrier fragment prior to digestion. In this way, even the removal of a methyl group from a single strand can be detected.

### Demethylation In Vitro

Following incubation in the presence of cell extract, a large percentage of the substrate DNA underwent demethylation and could then be cleaved with SmaI or HpaII (Figure 1). Several experiments strongly suggest that this demethylation is mediated by RNA molecules. First, when the extract is pretreated with relatively high concentrations (200–800 μg/ml) of proteinase K, the activity is not only preserved, but even augmented (see Figures 1B and 1C). Similar results were obtained using pronase E (300 μg/ml), and partial demethylation was seen when protease pretreatment was carried out in the presence of detergents (Figure 1C and legend). Enzyme activity was also maintained when the extract was preheated to 70°C (Figure 1B) or when the reaction itself took place at 65°C (data not shown). In contrast, incubation with RNase A, RNase $T_1$, or 10 mM EDTA completely abolished the activity (Figures 1B and 1D).

The extracts used in this reaction were prepared in buffer containing only Tris, KCl, and glycerol, and no additional potential substrates or sources of energy were added to the reaction mix. To test whether small molecule substrates may already be present endogenously in the crude cell extract, we carried out a simple purification step by isolating the void volume following G15 column chromatography. Despite this treatment, full demethylation activity was maintained, suggesting that the demodification reaction is mainly carried out by macromolecules and does not utilize free nucleotides as substrates or cofactors.

In the above experiments, the substrate was labeled exclusively at the HindIII site, permitting the detection of demethylation on only one strand of DNA. However, when the BamHI end of the molecule is also labeled, demodification yields stoichiometric amounts of the 580 and 230 bp fragments following SmaI digestion, indicating that both strands undergo this reaction at about the same rate (Figure 1E). As demethylation proceeds, the test site becomes fully unmodified and can then be cleaved with SmaI even without going through the denaturation and renaturation steps (data not shown). It thus appears that this enzymatic activity operates on both fully methylated and hemimethylated CpG sites, which is consistent with observations on demethylation in transiently transfected myoblasts (Paroush et al., 1990).

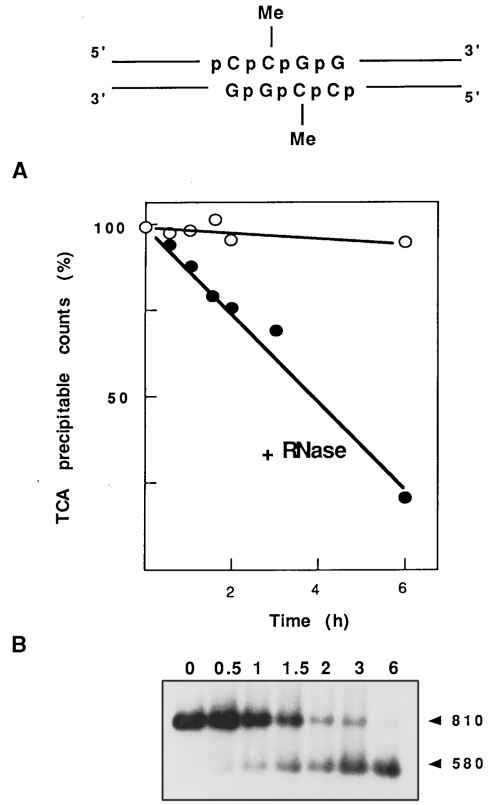


Figure 2. Conversion of Methyl Groups to an RNase-Sensitive Form

(A) The intact pBSα–act plasmid was in vitro methylated in the presence of [$^3$H]S-adenosylmethionine and then incubated with proteinase K–pretreated L8 cell extract. After various times, the reaction was stopped by the addition of 20 mM EDTA. Half of each sample was either treated (closed circles) or not treated (open circles) with 100 μg/ml RNase A at 37°C for 15 min, and then both halves were precipitated with TCA in the presence of 70 μg of herring sperm DNA. Similar results were obtained when samples were treated with 0.2 M NaOH at 60°C for 15 min instead of RNase A (data not shown). When the reaction was carried out in the presence of 10 mM EDTA, all of the counts remained TCA precipitable even after RNase A treatment (data not shown). The graph shows the percent of TCA-precipitable counts as compared with the time zero sample (~7000 cpm).

(B) An in vitro methylated HindIII end–labeled α-actin fragment (see Figure 1) was incubated in parallel with the same cell extract as in (A), and demethylation was followed by cleavage with SmaI. The same time scale applies to both (A) and (B).

### Transfer of Methyl Moieties to an RNase-Sensitive Form

To investigate the fate of methyl groups removed from the DNA during the course of the demethylation reaction, we labeled the α-actin plasmid (pBSα–act) with [$^3$H]methyl moieties at the HpaII sites and used it as a substrate in vitro. During the course of the reaction, which was monitored independently by following SmaI cleavage of an end-labeled fragment, methyl groups did not appear to be released from the DNA substrate as small molecules (Figure 2). Instead, the label continually remained trichloroacetic acid (TCA) precipitable throughout the course of the demethylation reaction. In light of the possible involvement of RNA molecules, we suspected that the methyl moieties may have been transferred from DNA to RNA. This idea was indeed strengthened by demonstrating that treatment of the product with either NaOH or RNase A resulted in the release of label into the TCA-soluble fraction. The conversion of methyl groups to a RNase-sensitive form appears to represent an integral part of the demethylation process, since both reactions are equally inhibited by EDTA or by prior treatment with RNase A (data not shown) and take place with similar kinetics (see Figure 2).

### Demethylation Is Carried out by Nucleotide Replacement

To define the methyl-containing compound that is actually removed from the substrate DNA during the course

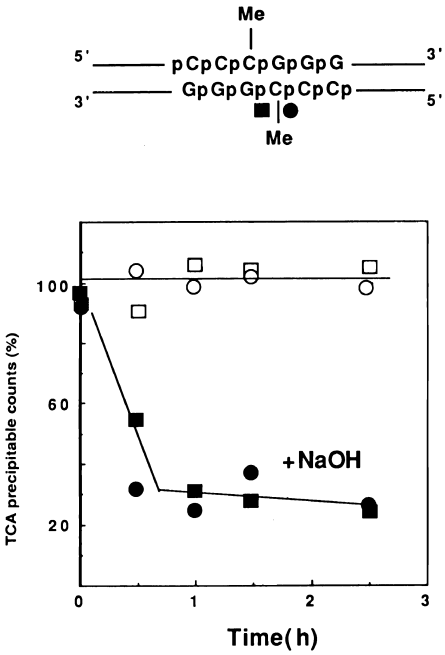


Figure 3. Conversion of Nucleotide Phosphates to a NaOH-Sensitive Form

In vitro methylated α-actin HindIII–BamHI fragments were prepared labeled specifically either at the 5′ phosphate of the methylated C nucleotide (circle) or the 5′ phosphate of the G nucleotide (square). Each substrate was incubated separately with the same proteinase K–pretreated L8 cell extract. At various times, samples were taken for TCA analysis as in Figure 2, either with (closed symbols) or without (open symbols) NaOH treatment. The results for both the C phosphate (circles) and the G phosphate (squares) are indicated. In control experiments, end-labeled substrate was assayed in the same manner. In this case, the radioactive counts were not converted to an RNase A– or NaOH-sensitive form.

of the reaction, we generated an upstream α-actin fragment labeled exclusively at the 5′ phosphate of the methylated cytosine within the SmaI site (see Figure 3). This was prepared by specifically end labeling the HpaII–XbaI restriction fragment (see Figure 1) and then using it as a primer on an M13 template to make a full-length α-actin molecule (see Experimental Procedures). When this DNA was treated with cell extract, the radioactive phosphate remained TCA precipitable, but was found to transfer to an NaOH- and RNase A–sensitive form in the same manner and with similar kinetics as the methyl moiety itself, suggesting that the entire nucleotide is removed from the DNA substrate.

We next asked whether additional nucleotide phosphates adjacent to the methylated cytosine may also be transferred during the course of the demethylation reaction. Using the same methodology, we specifically labeled the 5′ phosphate of the neighboring G nucleotide. This phosphate, as well, was removed from the DNA and converted into an NaOH-sensitive form. In fact, the 5′ phosphates of both the C and G nucleotides were transferred with similar kinetics (Figure 3). The availability of a substrate labeled with [$^{32}$P]dGTP enabled us to perform nearest-neighbor analysis on the demethylation reaction products. This demonstrated clearly that the adjacent C remained fully modified, suggesting that the 5-methylcytosine nucleotide together with its 3′ phosphate is maintained intact during the course of the reaction.

These internally labeled substrates were then used to obtain more direct evidence that nucleotides are indeed removed from the DNA as part of the demethylation reaction (see Figure 4). The α-actin fragment labeled specifically in the 5′ phosphate of the methylated C nucleotide (4, according to the numbering system shown in Figure 4A) was incubated with cell extract and then tested for demethylation. If this phosphate (4) were to remain in the DNA throughout the reaction, the resulting unmethylated product, when cut with SmaI, should yield a labeled 230 bp fragment. However, in the event that this nucleotide is indeed removed from the DNA, this fragment should lose its label. To provide an independent indicator of demethylation, we also included a HindIII end–labeled α-actin control fragment in the reaction mix. As shown in Figure 4B, this standard α-actin substrate clearly underwent demethylation and as a consequence became sensitive to SmaI cleavage, producing the expected labeled 580 bp fragment on the gel. However, the 3′ 230 bp digestion product, which should be labeled at the internal C nucleotide (4) on the same DNA strand, is clearly absent, strongly suggesting that the entire methylated cytosine nucleotide was removed from the DNA.

Analysis of an α-actin substrate specifically labeled at the 5′ phosphate of the G nucleotide (3) yielded similar results. Digestion with SmaI did not reveal the expected 580 bp fragment despite the fact that demethylation occurred normally, as indicated by the appearance of the smaller 230 bp fragment derived from a BamHI end–labeled control substrate (Figure 4C). Since both strands of the substrate undergo demethylation at about the same rate (Figure 1), these results show that the 5′ phosphate of the intact G nucleotide (3) is also removed during the course of the reaction.

To define better which nucleotide moieties within the SmaI site are actually involved in demethylation, we next tested the fate of the G (1 and 2) and C (5) nucleotide 5′ phosphates flanking the pCpG reaction center. For this purpose, we simultaneously labeled the phosphates in both cytosine residues (4 and 5) and used this substrate in the demethylation reaction. Despite the fact that the internal phosphate (4) is removed from the DNA, a labeled 230 bp fragment was obtained following SmaI digestion of the product, suggesting that the 5′ phosphate of the more external C nucleotide (5) remains in the DNA (Figure 4D). Molecules labeled in the 5′ phosphate groups of G residues 2 and 3 lose their radioactivity upon demethylation (data not shown), but when phosphates 1–5 were all labeled simultaneously in the same molecule, both the 580 bp and 230 bp fragments were visualized after digestion of the reaction product with SmaI, indirectly proving that the 5′ phosphate of the most external G nucleotide (1) is retained in the DNA (Figure 4E). Taken together, these studies indicate that both methylcytosine and the adjacent G nucleotide are removed from the DNA, and nearest-neighbor analysis of the reaction products (see above) suggests that this may occur as a dinucleotide unit.

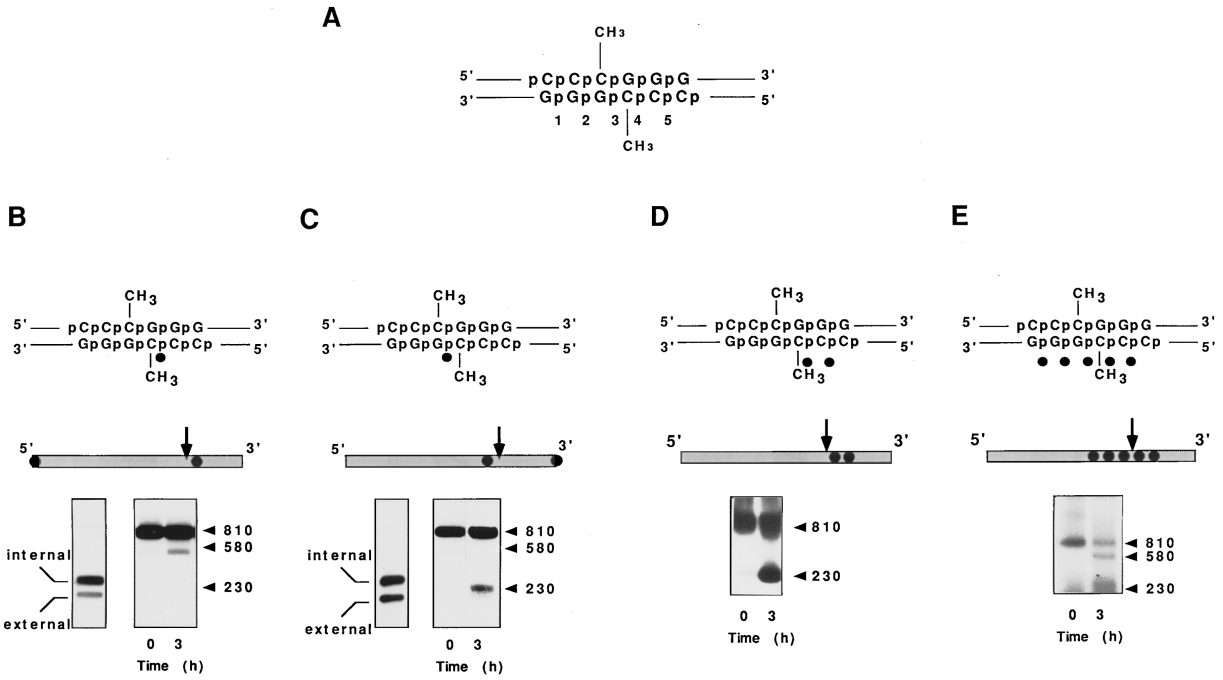


Figure 4. Removal of Phosphates from the DNA Substrate

The α-actin HindIII–BamHI fragment was labeled at a single specific nucleotide phosphate or at nucleotide phosphate groups within the SmaI site, methylated in vitro, incubated with proteinase K–pretreated extract, and tested for demethylation. Following denaturation and renaturation, the 810 bp fragment was gel purified and then used to test for cleavage with SmaI. In some of these experiments, an end labeled, in vitro methylated α-actin fragment is included in the reaction as a control for demethylation. For each test substrate, the labeled phosphates in the SmaI sequence are indicated by dots. An accompanying diagram shows the internal phosphate label relative to the SmaI cleavage site (arrow) and the position of the end label (dot) on the added control DNA. The internal labels are always on the lower strand.

(A) The nucleotide positions at the SmaI site that were labeled in these experiments have been numbered for convenient reference in the text.

(B) The α-actin substrate was labeled internally at the 5′ phosphate of the C nucleotide (4), and the control substrate was end labeled at the HindIII site. The unreacted mixture of methylated substrates was digested with SacI–BglII and electrophoresed to determine the ratio of internal (300 bp fragment) to external (170 bp fragment) label used in the demethylation reaction (see Figure 1 for the map positions of BglII and SacI). Following incubation with L8 extract for 0 or 3 hr, the reaction products were analyzed by SmaI digestion. Only the 580 bp fragment was still labeled, indicating that the internal phosphate had been removed during the course of the reaction.

(C) The α-actin substrate was labeled internally at the 5′ phosphate of the G nucleotide (3), and the control substrate was end labeled at the BamHI site. The unreacted mixture of methylated substrates was digested with SacI–BglII and electrophoresed to determine the ratio of internal (300 bp fragment) to external (130 bp fragment) label used in the demethylation reaction. Following incubation with L8 extract for 0 or 3 hr, the reaction products were analyzed by SmaI digestion. In this case, only the 230 bp fragment was still labeled, indicating that the internal phosphate had been removed during the course of the reaction. The same extract was employed for the experiments in both (B) and (C).

(D) The α-actin substrate was labeled internally at the 5′ phosphates of both C nucleotides (4 and 5). Demethylation was tested at 0 and 3 hr by digestion with SmaI, and the appearance of the 230 bp fragment indicated that the external C nucleotide (5) was retained during the course of the reaction. The product was also analyzed by digestion with HpaII, which cleaves between the two labeled C nucleotides. In this case, the 230 bp fragment containing the more external C nucleotide (5) was visualized, but the expected 60 bp fragment (see Figure 1A) containing the internal C nucleotide (4) was not (data not shown).

(E) The α-actin substrate was labeled internally at the 5′ nucleotide phosphate moieties (1–5). Demethylation was tested at 0 and 3 hr by digestion with SmaI, and the appearance of the 580 and 230 bp fragments indicated that both the external G nucleotide (1) and C nucleotide (5) were retained during the course of the reaction.

### Gene-Specific Demethylation

Studies in vivo and transfection experiments in tissue culture have shown that demethylation can be both tissue and stage specific. L8 myoblasts, for example, are able to carry out demethylation of an exogenously introduced muscle-specific α-actin gene, but are unable to perform this reaction when presented with other methylated sequences, such as the *Aprt* gene (Yisraeli et al., 1986). In contrast, F9 teratocarcinoma cells have been shown to direct the stage-specific demodification of CpG island DNA (Frank et al., 1991). Using the cell-free assay described above, we next tested whether this tissue specificity could also be reconstituted in vitro. To this end, L8 cell extracts were prepared and analyzed for their ability to demethylate two different sequences, the rat α-actin upstream region and the hamster *Aprt* gene. As expected, the α-actin sequence readily undergoes demodification in vitro. In contrast, a 3.2 kb EcoRI *Aprt* gene fragment remains heavily modified, even when exposed to the extract for extended times under the same conditions (Figure 5).

The specific demethylation events observed during normal development in vivo must be mediated through interactions between *cis*-acting elements and *trans*-acting protein factors. It is thus likely that the specific recognition events seen in vitro involve regulatory proteins

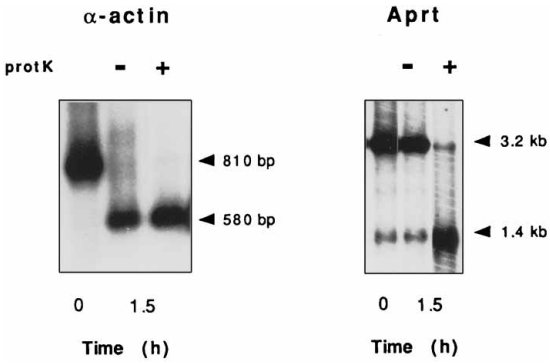


Figure 5. L8 Extract Sequence–Specific Demethylation

The 3.2 kb *Aprt* EcoRI fragment (see legend to Figure 6) was labeled at both ends, and the α-actin HindIII–BamHI fragment was labeled at the HindIII site. Both fragments were incubated separately with equal amounts of either untreated (minus) or proteinase K–pretreated (plus) L8 extract for 1.5 hr, and the degree of demethylation was measured by cleavage with SmaI (for α-actin) or HpaII (for *Aprt*). For comparison, the first lane of each experiment shows restriction digestion of the DNA at time zero, prior to incubation with extract. The results show that L8 extracts specifically demethylate the α-actin gene, but only when the extract has not been pretreated with proteinase K. Similar results were also obtained after a 3 hr or overnight incubation.

present in L8 extracts. To test whether this gene-specific demethylation indeed requires such protein factors, we assayed in vitro demethylation after treating the cell extract with proteinase K. Under these conditions, both the α-actin and *Aprt* gene fragments underwent demodification to approximately the same extent (Figure 5). It was previously demonstrated that gene-specific demethylation of α-actin in cell culture is directed by distinct *cis*-acting regulatory sequences located upstream of the gene coding region (Paroush et al., 1990). These same elements appear to be required for specific demethylation in vitro as well, since an α-actin construct carrying a deletion of these critical sequences fails to undergo demodification in the presence of L8 myoblast extracts (data not shown).

To evaluate the generality of this phenomenon, we analyzed cell extracts from F9 teratocarcinoma cells, which are known to contain a CpG island–specific demodification activity (Frank et al., 1991). As a first test of the F9 cell extracts, we compared in vitro demethylation on two separate fragments derived from the *Aprt* plasmid, a 3.2 kb fragment containing the CpG island and an adjacent 2.5 kb fragment made up entirely of nonisland sequences (Stein et al., 1982). The larger band clearly undergoes demodification, as shown by the fact

(plus) F9 extract for 3 hr, and the degree of demethylation was measured by cleavage with SmaI (for α-actin) or HpaII (for *Aprt*). For comparison, the first lane of each experiment shows restriction digestion of the DNA at time zero, prior to incubation with extract. The results show that F9 extracts specifically demethylate the *Aprt* CpG island–containing fragment, but only when the extract has not been pretreated with proteinase K. Identical results were also obtained after overnight incubation. Analysis of earlier timepoints indicated that the proteinase K–pretreated extract catalyzes demethylation of α-actin and *Aprt* with similar kinetics.

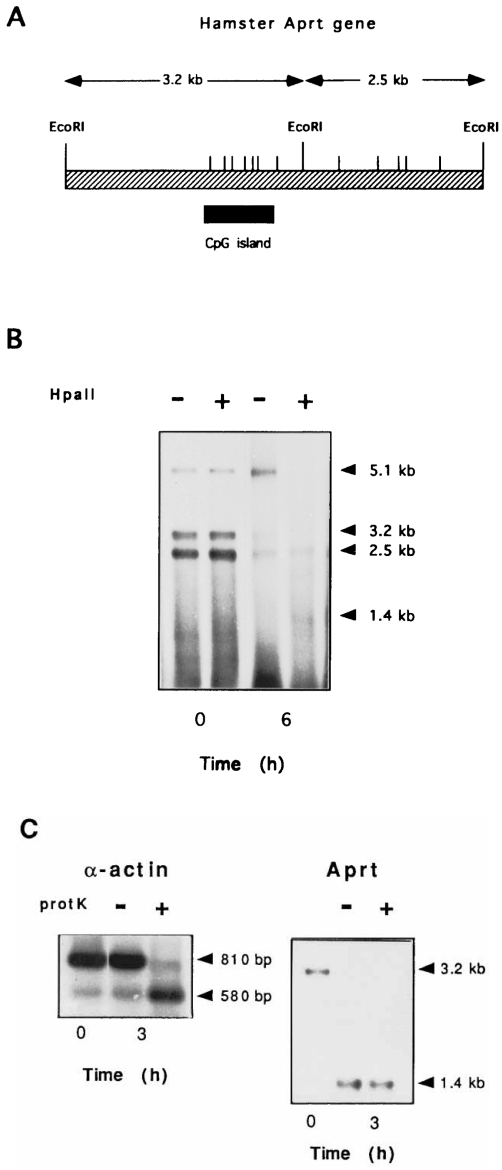


Figure 6. F9 Extract Sequence–Specific Demethylation

Fragments from the hamster *Aprt* gene or the α-actin gene were tested for demethylation using F9 cell extracts.

(A) Plasmid pHaprt yields three fragments when digested with EcoRI. The entire *Aprt* gene is included on two EcoRI fragments of 2.5 and 3.2 kb shown in the map. The 5′ end with its CpG island is located on the 3.2 kb fragment.

(B) pHaprt was in vitro methylated, end labeled, incubated with untreated F9 cell extract for various times, and assayed for demethylation by cleavage with HpaII. Undigested and HpaII-digested product are shown after 0 or 6 hr of reaction. In this experiment, all of the fragments have undergone some degradation, but the 3.2 kb band containing the *Aprt* CpG island has clearly undergone demethylation, producing the specific expected 1.4 kb fragment. In contrast, the 2.5 kb band remains fully modified and insensitive to HpaII digestion. The 5.1 kb fragment contains pBR 322 sequences, which are island like and undergo demethylation in F9 cells in vivo (unpublished data). This probably explains why it also undergoes demethylation in F9 extracts.

(C) The 3.2 kb *Aprt* EcoRI fragment was labeled at both ends, and the α-actin HindIII–BamHI fragment was labeled at the HindIII site. Both methylated, gel-purified fragments were incubated with equal amounts of either untreated (minus) or proteinase K–pretreated

that it can be cleaved with HpaII to yield a defined 1.4 kb product (Figure 6B). In contrast, the lower band remains relatively resistant to HpaII cleavage, indicating that it has not undergone demethylation. Untreated F9 extracts were also able to distinguish between this same 3.2 kb *Aprt* CpG island fragment, which underwent demethylation in vitro, and α-actin DNA, which did not (Figure 6C). However, following proteinase K treatment, this extract demodified both sequences with relatively equal efficiency. All of these data are consistent with the existence of a basic core demethylase activity capable of operating in a nondiscriminatory manner on a variety of different DNA sequences. Gene specificity is attained through interaction with appropriate protein factors. The basic demethylation machinery, although not always readily available, is probably present in a large number of different cell types. In keeping with this, we have been able to detect demodification activity in proteinase K–treated extracts from mouse L cells, the B-lymphocyte cell line S194, and from embryonic stem cells (data not shown).

## Discussion

Despite the apparent overall stability of DNA methylation patterns in somatic cells, the process of stage- and tissue-specific demethylation is still a prominent feature of development (Razin and Cedar, 1991). In fact, demodification represents the major mode for regulating cellular methylation patterns that are responsible for controlling basal gene expression in the organism. Despite their importance, however, the biochemical mechanism of these events has not yet been elucidated, and for this reason we developed an in vitro assay to study demodification.

### Demethylation Involves RNA

Using restriction enzymes to assay the presence of CpG methylation, we have shown that the entire process of demodification can be carried out in vitro by cell-free extracts in the absence of replication. Several key experimental observations support the suggestion that this reaction is mediated through the participation of RNA molecules. Both RNase A and RNase $T_1$ completely inhibit demethylation when added to cell extracts prior to the reaction. In contrast, demodification takes place even following vigorous protease treatment or when the extract was exposed to protein denaturation conditions (see legend to Figure 1). Further evidence supporting a role for RNA was obtained by showing that both the methyl moiety and phosphorylated nucleotides at the reaction center remain TCA precipitable, while being converted to a new form that is sensitive to both NaOH and RNase treatment. Taken together, these findings suggest that methylated nucleotides are actually transferred from DNA to as yet unidentified RNA molecules during the course of the demethylation reaction. We have not directly demonstrated the involvement of an RNA acceptor. However, when the demethylation products were analyzed by acrylamide gel electrophoresis, several RNase A–sensitive oligonucleotide bands were indeed observed (data not shown). In keeping with the proposed mechanism, these molecules appeared only when the methylated nucleotide phosphate was labeled, and not in reactions employing an end-labeled substrate.

### Biochemical Mechanism of Demethylation

Using single $^{32}P$ nucleotide–labeled substrates, we have been able to establish that the methyl group and the 5′ phosphates of the C and G nucleotide residues at the reaction center are removed from the DNA during the course of the reaction in vitro. It is likely that demethylation occurs by the same mechanism in vivo as well, since the phosphates were also excised following the transient transfection of these same $^{32}P$-labeled substrates into L8 myoblasts (data not shown). These results are also fully consistent with previous data on demethylation in differentiating erythroleukemia cells that indicated that the methylated cytosine residue itself is removed from the DNA during the course of the reaction (Razin et al., 1986).

Many details of the demethylation reaction are still not clear. For example, we have not yet fully characterized the unmethylated DNA product. This reacted DNA does appear to retain its sequence integrity, since demethylated sites can still be cleaved with a number of different restriction enzymes, such as HpaII, MspI, and SmaI, even after the reaction has taken place. In fact, the assay itself is dependent on this property. Furthermore, the demethylated DNA itself does not seem to contain nicks at the site of reaction, since an end-labeled product remains intact on a denaturing gel (unpublished data) and forms full-length double-stranded molecules after reannealing to excess unlabeled DNA. This product was also insensitive to cleavage with NaOH at the site of demethylation, suggesting that the replacement is carried out with deoxyribonucleotides rather than ribonucleotides. Since the reaction mix was not supplemented with nucleotide substrates, and since repair synthesis is unlikely to occur following pretreatment with proteinase K, we propose that these unmethylated nucleotide replacements are derived from a macromolecule, perhaps associated with the same entity involved in the demethylation reaction itself.

Taken together, these studies indicate that demethylation takes place through a process of nucleotide replacement utilizing cleavage and ligation steps, and this is consistent with the possibility that the reaction is catalyzed either by a ribozyme or by some form of enzymatically active RNA–protein complex. Both group I ribozymes (Herschlag and Cech, 1990; Robertson and Joyce, 1990) and ribonuclease P (Smith and Pace, 1993) can be made to cleave single-stranded DNA in vitro, and group II ribozymes can even carry out DNA–RNA ligation (Morl et al., 1992). Furthermore, it was recently demonstrated that a group II intron RNA serves as the catalytic component of a natural DNA endonuclease that cleaves only one strand of a DNA duplex in a site-specific manner, and this may represent a natural ribozyme-type reaction actually dedicated to operate on a DNA substrate (Zimmerly et al., 1995; see also Griffin et al., 1995). While these studies indicate that ribozymes have the potential to catalyze the same chemical reactions

that may be involved in demethylation, direct proof for this model will have to await isolation and purification of the enzymatically active molecules and their characterization in vitro.

In vitro demethylation has recently been observed both in chicken embryo extracts (Jost, 1993) and tissue culture cells (Vairapandi and Duker, 1993). Using a highly purified enzyme preparation, it has now been shown (Jost et al., 1995) that this demethylation reaction is initiated by a glycosylase that removes the methylated base. This site is probably then nicked by means of an apyrimidinic acid–specific endonuclease, which in turn induces DNA repair. This demethylation reaction appears to be non–sequence specific and prefers hemimethylated DNA substrates. Furthermore, the enzyme itself copurifies together with a distinct G-T mismatch glycosylase activity, raising questions as to what the actual role of this enzyme might be in vivo.

### Regulation and Specificity of Demethylation

The demethylation that we have detected in L8 or F9 extracts is gene specific, making it very likely that this reaction mimics the process that occurs in vivo. The presence of proteins appears to have a dual influence on the demodification reaction, and treatment of extracts with proteinase K causes both stimulation of enzyme activity and a loss of specificity. On this basis, it is reasonable to suggest that cell extracts contain protein factors that normally inhibit the demethylation activity. Proteins also seem to be required to direct gene-specific demethylation in extracts from different cell types.

On the basis of these observations, one can conceive of a single activity accommodating the various different demethylation events that occur during normal development. The basic machinery needed for demethylation is probably present in most, if not all, cell types during development, but may be kept in an inactive state through interactions with protein inhibitors. We propose that there are two independent pathways for stimulating enzymatic demethylation activity (see Figure 7). One mechanism would operate through the destruction or modification of a bound protein inhibitor, thus releasing it from the demethylation complex and bringing about activation of the enzyme. In vivo, this process may take place in the early preimplantation embryo, where one observes a generalized and relatively nonspecific demodification throughout the genome (Kafri et al., 1993). This phenomenon is indeed very similar both quantitatively and qualitatively to what is observed in vitro when the inhibitory proteins are artificially destroyed by proteinase K.

A second form of demethylation is gene or region specific. This type of activity is first seen at the time of implantation, when almost the entire genome undergoes de novo methylation and only CpG island sequences are protected from this process, presumably through specific demethylation events. We suggest that the same basic demethylase activity is involved at this stage, as well, but that its activation is brought about through interactions with specific proteins anchored at the sites of CpG islands. In this model, demethylation would occur only as long as the active species is tethered to this specific locus on the DNA and only within the defined region having access to the demethylation activity. It has already been demonstrated that Sp1 elements are present in almost all CpG islands (Holler et al., 1988) and that this *cis*-acting element may be essential for protecting the islands from de novo modification at the time of implantation in vivo (Brandeis et al., 1994; Macleod et al., 1994). Stage-specific Sp1-related proteins could thus play a role in attracting and activating the demethylase complex and consequently causing island undermethylation at this critical point in embryogenesis.

At later stages of development, demethylation takes place in a gene-specific manner concomitant with the process of differentiation. Here as well, the removal of methyl groups appears to be directed by *cis*-acting elements associated with the gene. In the case of the mouse κ gene, the presence of the κ-intronic enhancer and the nearby MAR element is required for demethylation to take place, and it is likely that this is mediated through protein factors that interact with these sequences in a cell type–specific manner (Lichtenstein et al., 1994). Preliminary evidence already suggests that NF-κB itself plays a role in demethylation (Kirillov et al., 1996), but additional factors may also be required. These proteins could serve as the basis for attracting the demethylase to the κ gene and simultaneously activating

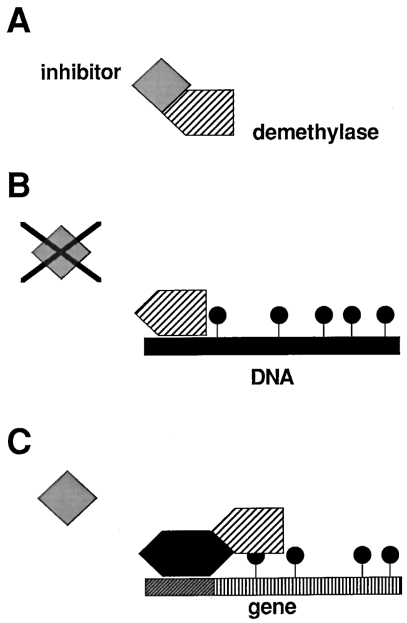


Figure 7. A Unified Model for Demethylation In Vivo

(A) The basic demethylase probably contains an RNA component and is present in all cells during development, but is prevented from operating on DNA by interactions with a protein inhibitor.

(B) In the preimplantation embryo, the inhibitor is either degraded or modified so that it is released from the demethylase, which is now free to remove the methyl groups (closed circles) from the entire genome in a non-gene-specific manner.

(C) Gene-specific demethylation takes place when *trans*-acting factors (closed hexagon) are bound to *cis*-acting elements in the vicinity of the gene. These proteins can interact with the demethylase complex and release it from the inhibitor, allowing demodification of DNA within reach of the anchored enzyme.

it at the proper time in development. Once tethered to this particular locus, it would direct demethylation in the DNA region surrounding the gene. A detailed analysis of this particular gene shows that undermethylation in B cells is regional and independent of sequence context (unpublished data), and this is consistent with the proposed mechanism. Other tissue-specific genes are also undermethylated in this same patch-like pattern (Yeivin and Razin, 1993), suggesting that they may also undergo demethylation by this mode of action.

## Experimental Procedures

### Cell Growth

Mononucleated cells of the rat myogenic line L8 (Yaffe and Saxel, 1977) were grown in Waymouth's MB 751/1 medium supplemented with 15% fetal calf serum, 2 mM glutamine, 1000 U/ml penicillin, and 100 μg/ml streptomycin. F9 teratocarcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1000 U/ml penicillin, and 100 μg/ml streptomycin.

### Preparation of Whole-Cell Extract

Demethylation activity was maximally observed in extracts derived from cultures at near confluence. These cells were trypsinized, collected, washed twice with phosphate-buffered saline, and then suspended ($10^7$ cells per 100 μl) in cell extract buffer (CE) containing 20 mM Tris–HCl (pH 7.5), 0.4 M KCl, 20% (v/v) glycerol, 2 mM DTT and frozen immediately at −80°C for 1 hr. Thawing was done by centrifuging at 10,000 × g for 15 min at 4°C. The whole-cell extract supernatant was diluted with 3 vol (L8 cells) or 10 vol (F9 cells) of water. For many experiments, the cell extract was treated with 200 μg/ml proteinase K for 1 hr at 56°C. To test the effect of RNA digestion on the demethylation reaction, the diluted extract was treated with either 100 μg/ml RNase A (Sigma), 30 U/ml bovine pancreatic RNase mix (Boehringer Mannheim), or 1000 U/ml RNase $T_1$ (Boehringer Mannheim) for 1 hr at 37°C prior to proteinase K treatment. For some experiments, the thawed whole-cell extract supernatant was loaded on a 2 ml G-15 Sephadex (Pharmacia) column equilibrated with CE buffer. The column was spun at 1100 rpm for 1 min at room temperature, and the resulting void volume was then diluted with water as described above.

The demethylation potential of these cell extracts was found to be unstable when stored either concentrated or after diluting for short time periods of 1–3 days even at −70°C. For this reason, each extract was prepared freshly prior to use. In addition, the activity of each individual extract was variable, with some preparations yielding 100% demethylation within 1 hr under standard reaction conditions, while in others only partial demethylation was attained after overnight incubation. Attempts to solve these problems by adding RNasin to prevent RNA degradation were not successful. Both the untreated and proteinase K–pretreated extracts revealed variable degrees of DNA degradation, and this can be seen in some of the experiments as a decrease in the amount of initial DNA substrate with time.

### Demethylation Substrates

Methylation in vitro was carried out on 20–50 μg of whole plasmid or purified restriction fragment by incubating with HpaII methylase (Fermentas; 5 U per microgram of DNA) overnight at 37°C in the following reaction mixture: 25 mM Tris–HCl (pH 7.5), 20 mM NaCl, 0.1 mg/ml BSA, 10 mM EDTA, 5 mM DTT, and 160 μM S-adenosylmethionine. The reaction was terminated by incubation for 1 hr at 56°C in the presence of 200 μg/ml proteinase K, extracted with phenol:chloroform (1:1), and precipitated with ethanol. [$^3$H]methyl substrates were prepared in the same way, using 5 μg of DNA and 15 μM [methyl-$^3$H]methionine (Amersham; 71 Ci/mmol).

The plasmid pBSα–act contains an 809 bp EcoRI–BstEII α-actin upstream fragment inserted into the pBluescript KS(−) vector (Paroush et al., 1990). For the standard assay, this plasmid was HpaII methylated in vitro, cleaved with BamHI or HindIII, end labeled, cut again with the second enzyme, and electrophoresed on an agarose gel, and the correct upstream HindIII–BamHI fragment was then extracted and purified by Geneclean II (Bio101). For some experiments, the hamster pHaprt plasmid (Lowy et al., 1980) was HpaII methylated, digested with EcoRI, and end labeled. The 3.2 kb fragment containing the *Aprt* 5′ CpG island was analyzed for demethylation, either alone or together with the other EcoRI fragments derived from this plasmid.

To obtain α-actin substrates labeled at specific nucleotide phosphates within the SmaI site, we carried out second strand synthesis on an M13mp19 construct containing the HindIII–XbaI fragment from pBSα–act (see Figure 1). mp19 was chosen so that the upper strand is the template (see Figure 4) and the lower strand is filled in by DNA synthesis. To label the 5′ phosphate of either the internal G (3) or C (4) nucleotides in the SmaI site (see Figure 4), we used a gel-purified 230 bp HpaII–XbaI fragment from pBSα–act as the primer. This fragment contains a 5′ CG overhang at the HpaII site, which was labeled with either [$^{32}$P]dCTP or [$^{32}$P]dGTP. The gel-purified, labeled fragment was then denatured and rehybridized to the M13 template, generating a substrate for second strand synthesis (Keshet et al., 1985), which was carried out with nonradioactive nucleotides. The product of this reaction was methylated in vitro and cleaved with HindIII–BamHI, and the α-actin upstream band was then purified from an agarose gel before being used in the demethylation reaction, usually after mixing it with end-labeled methylated fragment carrying an approximately equivalent amount of radioactive label.

A similar strategy was used to obtain α-actin substrates labeled specifically at the 5′ phosphates of both C nucleotides (4 and 5) or both G nucleotides (2 and 3) within the SmaI site. In this case, the labeled primer was generated from the 230 bp XmaI–XbaI fragment (see Figure 1). XmaI is an isoschizomer of SmaI, but leaves a 5′ CCGG overhang, which could be labeled with either [$^{32}$P]dCTP or [$^{32}$P]dGTP. The final substrate was then generated by second strand synthesis with nonlabeled nucleotides, methylation, and purification, as described above. To label nucleotide phosphates 1–5 (Figure 4E), we annealed the HpaII–XbaI primer fragment to the M13 template and initiated synthesis with [$^{32}$P]dCTP and [$^{32}$P]GTP. The free-labeled nucleotide triphosphates were removed by two G50 purification steps, and second strand synthesis was then continued with unlabeled nucleotides. The final methylated substrate was prepared as described above.

### Demethylation Assay

Labeled, methylated DNA (3–30 ng) was incubated in 200 μl of whole-cell extract (7 mM Tris–HCl [pH 7.5], 0.13 M KCl, 7% [v/v] glycerol, 0.7 mM DTT) at 32°C. At various timepoints, the reaction was terminated by adding NaCl to a final concentration of 0.3 M and an approximate 20-fold molar excess of the equivalent unmethylated, unlabeled DNA. The DNA product was then extracted with phenol:chloroform (1:1), ethanol precipitated, denatured for 2 min at 100°C in the presence of 1 mM EDTA, adjusted to 50 mM NaCl, and renatured 1.5 hr at 60°C. The resulting labeled DNA was then treated with the restriction enzymes SmaI or HpaII, electrophoresed on 1% agarose gels, and autoradiographed to evaluate the extent of demethylation. Nearest-neighbor analysis was perfomed on the [$^{32}$P]dGTP SmaI site–labeled substrate and on its reaction products (Gruenbaum et al., 1981).

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